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Salmonella spp., *Vibrio* spp., *Clostridium perfringens*, and *Plesiomonas shigelloides* in marine and freshwater invertebrates from coastal California ecosystems.

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2   and freshwater invertebrates from coastal California ecosystems.

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21   Running head: Coastal invertebrate bacterial ecology

22

22 ***Abstract***

23   The coastal ecosystems of California are highly utilized by humans and animals, but the

24 ecology of fecal bacteria at the land-sea interface is not well understood. This study evaluated

25 the distribution of potentially pathogenic bacteria in invertebrates from linked marine, estuarine,

26 and freshwater ecosystems in central California. A variety of filter-feeding clams, mussels,

27 worms, and crab tissues were selectively cultured for *Salmonella* spp., *Campylobacter* spp.,

28 *Escherichia coli*-O157, *Clostridium perfringens, Plesiomonas shigelloides*, and *Vibrio* spp. A

29 longitudinal study assessed environmental risk factors for detecting these bacterial species in

30 sentinel mussel batches. Putative risk factors included mussel collection near higher risk areas

31 for livestock or human sewage exposure, adjacent human population density, season, recent

32 precipitation, water temperature, water type, bivalve type, and freshwater outflow exposure.

33 Bacteria detected in invertebrates included *Salmonella* spp., *Clostridium perfringens,*

34 *Plesiomonas shigelloides, Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio alginolyticus.*

35 Overall, 80% of mussel batches were culture-positive for at least one of the bacterial species,

36 though the pathogens *Campylobacter, E. coli*-O157, and *Salmonella* were not detected. Many of

37 the same bacterial species were also cultured from upstream estuarine and riverine invertebrates.

38 Exposure to human sewage sources, recent precipitation, and water temperature were significant

39 risk factors for bacterial detection in sentinel mussel batches. These findings are consistent with

40 the hypothesis that filter-feeding invertebrates along the coast concentrate fecal bacteria flowing

41 from land to sea, and show that the relationships between anthropogenic effects on coastal

42 ecosystems and the environmental niches of fecal bacteria are complex and dynamic

43

43   *Introduction*

44   The ecology of fecal bacteria entering nearshore aquatic environments along the California

45   coast is not well understood, but is gaining in importance as human populations utilizing these

46   ecosystems grow (8, 37, 40, 43). Environmental loading of fecal byproducts from humans and

47   their associated animals is significant and can affect the quality of water and food resources in

48   coastal ecosystems (13, 24). Terrestrial sources of fecal waste entering aquatic ecosystems

49   include animal-derived fecal matter carried in storm runoff, and human sources such as sewage

50   outfalls, leaky septic tanks, and boat discharges. Many of the fecal parasites, bacteria, and

51   viruses that have been detected in freshwater, estuarine, and marine environments are potential

52   pathogens to humans and animals, and their environmental niches are quite diverse (6, 36).

53   Our previous studies have investigated the epidemiology of the fecal protozoan parasites

54   *Cryptosporidium, Giardia,* and *Toxoplasma* species in coastal California ecosystems (8, 28-30,

55   32). All of these parasite life cycles involve fragile zoite stages that develop in the digestive

56   tracts of animal hosts, including in some cases humans, and sturdy oocyst or cyst stages that are

57   shed in the feces and can remain viable in the environment for prolonged periods of time (12, 39).

58   Investigation into the ecology of these parasites in aquatic ecosystems revealed that detection of

59   *Cryptosporidium* and *Giardia* spp. in bivalve shellfish was associated with exposure to

60   freshwater sources and was greatest during the wet season in California (30, 32). *Toxoplasma*

61   *gondii* in a marine mammal, the Southern sea otter (*Enhydra lutris nereis*), has also been

62   correlated with exposure to areas of high freshwater outflow into the nearshore marine

63   environment (28). While the life cycles of fecal bacteria are quite different than fecal protozoa,

64   some of the same risk factors may apply to the transport of fecal bacteria from land to sea.

65   Fecal bacteria entering aquatic ecosystems in coastal California may have a more complex

66   ecological niche because unlike the protozoans that are only known to multiply within animal

67   cells, bacteria can multiply in the environment as well as within animal hosts (18, 44). This

68   suggests that once fecal bacteria enter the aquatic environment, they may be able to establish

69   long-term reservoirs, depending on each species' ability to acclimate and tolerate a range of

70   environmental conditions. The bacteria in this study include primarily terrestrial fecal bacteria

71   such as *Salmonella* and *Campylobacter* spp., as well as fecal bacteria well-adapted to aquatic

72   environments such as *Vibrio* and *Plesiomonas* spp., all of which can act as pathogens under the

73   appropriate host, microbe, and environmental conditions (2, 5, 18, 40, 44).

74   Filter-feeding invertebrates play an important role in the ecology of aquatic pathogens

75   because they concentrate microbes from the surrounding waters (4, 16, 27). As a result, filter-

76   feeders can act as bioindicators of the microbial diversity present in aquatic ecosystems, as well

77   as food items that provide concentrated microbial doses to the animals and humans that consume

78   them. The goal of this study was to investigate the distribution of potentially pathogenic bacteria

79   among aquatic invertebrates in marine, estuarine, and freshwater ecosystems along the central

80   California coast. Our primary hypothesis was that if fecal pollution is flowing from land to sea,

81   then detection of fecal bacteria in invertebrates may be greatest near sites designated as higher

82   risk for livestock runoff or human sewage exposure, compared to sites designated as lower risk

83   for these sources. Based on our previous protozoal studies, we also hypothesized that high

84   freshwater outflow exposure and wet season sampling would be associated with greater detection

85   of fecal bacteria in bivalves.

86

87   *Methods*

88   *Study Design.*   Bacteria selected for this study included *Salmonella* spp., *Campylobacter*

89   spp., *Escherichia coli*-O157, *Clostridium perfringens*, *Vibrio* spp., and *Plesiomonas shigelloides.*

90   Three approaches were taken to evaluating their distribution among invertebrates along the

91   central California coast. First, a longitudinal study was set up to deploy and collect sentinel

92   mussel batches for quarterly testing during the dry (June-November) and wet (December-May)

93   seasons along the central California coast. Figure 1 shows the various mussel sites sampled over

94   the course of the three year study, 2001-2004. Mussels were initially collected from a pristine

95   site near Bodega Bay, California, from which the target bacteria were not detected at any point

96   during the study, and were deployed at three sites considered at higher risk for human sewage

97   exposure, three sites at higher risk for livestock runoff, and three sites distant to these fecal

98   sources (32). Mussels were collected quarterly as batches of 30 and when transplanted mussels

99   were not available, resident mussels from the study sites were collected instead. Data on

100   environmental risk factors that could be associated with fecal pathogen pollution flowing from

101   terrestrial to aquatic ecosystems were also collected for each study site and sampling time.

102   Second, a comparative study was conducted of multiple invertebrate species collected from

103   muddy and sandy habitats near Moss Landing (Monterey Bay) and Morro Bay, California.

104   Clams, innkeeper worms, and mussels were sampled from a muddy estuarine habitat on the same

105   day that clams, sand crabs, and mussels were collected from a nearby sandy habitat just outside

106   the estuary during the wet season of 2002-2003. The target sample size was again 30 per species.

107   Third, freshwater clams (*Corbicula fluminea*) were deployed in the San Lorenzo and Salinas

108   Rivers, two rivers with high historic coliform counts along the central coast (http://www.water

109   boards.ca.gov/centralcoast/BasinPlan/ Documents/3BactiObjsStaffReport05-04-04.doc), both of

110   which feed into the Monterey Bay as shown in Figure 1. Clams were collected in batches of 30,

111   once during the dry season and again during the wet season for two consecutive years, 2002-

112   2004. Bivalve collection and deployment methods were in accordance with long-term coastal

113   monitoring protocols (30).

114

115   *Invertebrate Sampling.*   Surf mussels (*Mytilus californianus*) and bay mussels (*Mytilus*

116   *edulis*) were used in the longitudinal mussel study. Invertebrate species sampled from the

117   estuaries near Moss Landing and Morro Bay, CA included fat innkeeper worms (*Urechis caupo*),

118   gaper clams (*Tresus nuttalli*), Washington clams (*Saxidomus nuttalli*), bent-nose clams (*Macoma*

119   spp.), and mussels (*Mytilus* spp.). Sampling from the sandy habitats outside the estuaries

120   included Pismo clams (*Tivela stultorum*), mussels (*Mytilus* spp.) and two types of sand crabs

121   (*Emerita analoga* and *Blepharipoda occidentalis*). Freshwater clams (*Corbicula fluminea*) were

122   deployed for invertebrate testing in the San Lorenzo and Salinas Rivers.

123   Invertebrates were collected as batches of 30, of which six individuals were pooled per

124   culture batch from each site and timepoint. Invertebrates were transported on ice to laboratories

125   at the University of California, Davis (UCD), or to the Marine Wildlife Veterinary Care and

126   Research Center in Santa Cruz, CA, for dissection within 24 hours of collection. Hemolymph,

127   gill, and digestive gland tissues were collected as described (30, 32), and cultured for bacteria

128   using selective media at UCD. The best invertebrate tissue to culture was determined by

129   comparing the results of culturing hemolymph, gill and digestive gland tissues from the same

130   invertebrates in the first year, with only digestive gland cultured in subsequent years.

131

132   *Bacterial Culture.* A 1-2 g aliquot of invertebrate tissues was macerated with a sterile swab

133   and saline in a microcentrifuge tube before plating onto selective media according to established

134   protocols (34). Cultures were enriched for *Salmonella* and *Vibrio* spp. for 24 hrs using selenite

135   broth and alkaline peptone water before plating onto Xylose Lactose Tergitol 4 (XLT4) and

136   Thiosulfate Citrate Bile Sucrose (TCBS) agar, respectively. *Campylobacter* cultures utilized

137   selective media containing Cefoperazone Vancomycin and Amphotericin B (Campy CVA) in a

138   microaerophilic environment. *Escherichia coli*-O157 cultures were performed on Cefixime

139   Potassium Tellurite Sorbitol MacConkey (CT SMAC) plates. *Clostridium perfringens* was

140   identified using Egg Yolk Agar (EYA) under anaerobic conditions. *Plesiomonas* was identified

141   from MacConkey plates. All incubations were performed at 37°C. Morphologic appearance and

142   biochemical testing was used to initially identify the isolates, with further characterization using

143   specific antiserum, API 20E strips, serotyping, and sequence analysis of the 16S rRNA gene.

144

145   *Risk Factors.*   Environmental factors that could be associated with bacterial detection in

146   mussels were identified and the data assimilated for all study sites and timepoints. Categorical

147   data on fecal risk category was defined as lower risk for fecal pollution if sites were more than 5

148   km away from known fecal sources, as higher risk for livestock fecal sources if sites were less

149   than 5 km from livestock runoff, and as higher risk for human sewage exposure if sites were less

150   than 5 km from a sewage outfall or historic septic leakage. The distance of 5 km was selected

151   based on biologic plausibility in a previous study (32) and in consultation with local resource

152   managers who identified known fecal input sources within a 5 km radius of the sampling site.

153   Season was categorized by dry (June-November) or wet (December-May) season for each

154   collection. Recent precipitation was recorded as present or absent for the past day and past week.

155   Water type at each collection site was defined as estuarine or marine. Bivalve type was recorded

156   as resident or transplanted for each mussel batch. Freshwater outflow exposure data, defined as

157   low = <10 million L, medium = 10-100 million L, high = >100 million L of freshwater exposure

158   in the past day, were available for each study site and mussel collection date from our previous

159   *Cryptosporidium* study (32). In addition, human densities of the adjacent coastline were

160   estimated using 2000 census data (http://www.census.gov/main/www/ cen2000.html) and water

161   temperatures for each mussel collection date were obtained from National Oceanic and

162   Atmospheric Administration records (http://co-ops. nos.noaa.gov/).

163

164   *Statistical Analysis.*   McNemar's χ^2 test for paired data was used to evaluate whether

165   significant differences in culture success could be detected among hemolymph, gill, and

166   digestive tissues from the same bivalves, as well as comparing culture results from mud and sand

167   invertebrates collected at the same estuaries. For the mussel risk factor analysis, univariate and

168   then multivariate logistic regression models were created to quantify the strength of association

169   between environmental variables and detection of the enteric bacteria of interest. Logistic

170   regression is a powerful tool for simultaneously assessing the relative contributions of multiple

171   risk factors that may be associated with a dichotomous outcome of interest such as the presence

172   or absence of a bacterial species. The odds ratio produced for each risk factor is interpreted as

173   the probability that the outcome of interest will occur in samples with the risk factor, relative to

174   the probability of the outcome occurring in samples without the risk factor (or referent category).

175   To minimize sparse data problems, models were only created for bacteria that were detected

176   more than five times over the three year study (19). No study sites were within 5 km of each

177   other, and only one site (the most downstream site) per estuary was included. A cluster effect

178   was used in all logistic regression models, to adjust for repeated sampling at the same sites over

179   time. All statistical analyses were performed using Stata software (Stata Corp, College Station,

180   TX, USA). Significant P values were defined as P<0.1.

181

182   ***Results***

183   *Tissue Comparison Study.*   During the first year of bivalve collections, the hemolymph,

184   gill, and digestive gland tissues from the same animals (n=110) were cultured to determine the

185   optimal tissue for long-term study. There was no significant difference between the frequency of

186   isolation of enteric bacteria from hemolymph or gill tissues (P=0.4). However, digestive gland

187   tissues significantly outperformed both hemolymph and gill tissues (P<0.001). Based on these

188   results, only digestive gland was cultured for the remainder of the study.

189

190   *Mussel Study.* The three year mussel study involved deploying and collecting mussels quarterly

191   from sites ranging south from Bodega Bay to Morro Bay, CA as shown in Figure 1. Forty-six

192   mussel batches met the study inclusion criteria for statistical analysis, with each study site

193   sampled at least three times during the study. Table 1 shows the bacterial prevalence among the

194   46 batches in the dry season, wet season, and in total. Overall, 80% of mussel batches during the

195   dry and wet seasons were positive for at least one of the bacterial species, though no

196   *Campylobacter, E.coli*-O157, or *Salmonella* spp. were detected. *Plesiomonas shigelloides* was

197   detected in 4% of batches collected during the dry season but not during the wet season. *Vibrio*

198   *parahaemolyticus, V. cholerae* non-O1, and *V. alginolyticus* were detected in up to 48% of

199   mussel batches collected during the dry season and up to 26% of mussel batches collected during

200   the wet season. In contrast, *C. perfringens* was detected more often in the wet season (68%) than

201   in the dry season (41%).

202   Univariate analyses evaluated the strength of association between individual environmental

203   variables and a mussel batch culturing positive for each bacterial group. The odds ratios and

204   associated P values for factors significantly associated with detection of *V. cholerae, V.*

205   *alginolyticus, C. perfringens*, and 'Any bacterial species' outcomes are given in Table 2. The

206   other individual bacterial species were not included due to the sparse nature of the data. *Vibrio*

207   *cholerae* was not significantly associated with any of the environmental variables. A mussel

208   batch was significantly less likely to test positive for *V. alginolyticus* during the wet season or if

209   there was precipitation in the day or week preceding mussel collection. A batch was more likely

210   to have *V. alginolyticus* isolated if the water temperature was greater than 12°C at the time of

211   sampling or if the study site was marine as compared to estuarine. Detecting *C. perfringens* in

212   mussel batches was significantly more likely to occur during the wet season, if there was not

213   precipitation in the day preceding mussel collection, or if the water temperature was less than

214   12°C. Mussel batches in which any of the bacteria were cultured were significantly associated

215   with the higher risk category for exposure to human sewage, and with not having precipitation in

216   the day or week preceding mussel collections. Human density and bivalve type were not

217   significantly associated with any of the bacterial outcomes.

218   Forward-stepping multivariate logistic regression was used to examine multiple

219   environmental variables simultaneously against the outcome of a batch culturing positive for the

220   bacteria of interest. Table 3 shows the three models for which significant explanatory variables

221   were found. Culturing *V. alginolyticus* from mussel batches collected within 24 hours of a

222   precipitation event was 5 times less likely (odds ratio = 0.2) than mussel batches in which no

223   precipitation occurred in the preceding day (P<0.01). *Clostridium perfringens* was also less

224   likely to be detected in mussel batches collected within a day following a precipitation event

225   (P=0.07), and was associated with water temperatures less than 12°C (P=0.02). Detecting any of

226   the targeted bacterial species in a mussel batch was significantly associated with no precipitation

227   in the day preceding mussel collection (P<0.01), and was 39 times greater if the mussel batch

228   was collected from a site considered higher risk for human sewage exposure compared to sites

229   considered lower risk for human sewage or livestock waste exposure (P=0.01).

230

231   *Comparative Invertebrate Study.* The results of culturing pools of six invertebrates from

232   muddy and sandy habitats near two fecal-impacted estuaries in the wet season of 2002-2003 are

233   shown in Table 4. Bacterial pathogens were detected in the innkeeper worms, bivalves, and sand

234   crabs that live in the sediment, as well as in mussels that were suspended higher up in the water

235   column. *Salmonella* Typhimurium was detected in Pismo clams and *S.* Heidelberg in fat

236   innkeeper worms at the Moss Landing site. *Vibrio parahaemolyticus* was cultured from Pismo

237   clams and from at the Morro Bay site. *Vibrio alginolyticus* and *Clostridium perfringens* were

238   detected in a variety of invertebrate species at both sites. Invertebrate pools (n=44) from the

239   muddy sites inside the estuaries were more often positive for enteric bacteria than invertebrates

240   collected at the sandy sites just outside the estuaries (P<0.01).

241

242   *Freshwater Clam Study.* The prevalence of each bacterial species cultured from pools of six

243   freshwater clams collected during the 2002-2004 dry and wet seasons is shown in Table 5.

244   *Vibrio cholerae V. alginolyticus,* and *C. perfringens* were only detected during the dry seasons.

245   Downstream clam batches were more often culture-positive for bacteria than upstream batches.

246   No *Campylobacter, E.coli*-O157, *Plesiomonas, Salmonella,* or other *Vibrio* spp. were detected.

247   During the first wet season sampling, no clams were found alive at the lower San Lorenzo River

248 site and both Salinas River sites. Clams were repeatedly deployed at a third Salinas River site but

249 the clam batches were consistently missing at each sampling timepoint. Sites and time periods

250 for which no clams were cultured were excluded from further analysis.

251

252 ***Discussion***

253 The goal of this study was to undertake the first multiyear investigation of the distribution of

254 potentially pathogenic bacteria among aquatic invertebrates in linked marine, estuarine, and

255 freshwater ecosystems along the Pacific coast of North America. *Campylobacter* and *E.coli-*

256 O157 were not detected in the study, *Salmonella* and *Plesiomonas shigelloides* were only rarely

257 detected, and *Clostridium perfringens* and *Vibrio* spp. were commonly detected. We had

258 hypothesized that if biologic pollution due to fecal bacteria is flowing from land to sea, then

259 detection of these organisms in invertebrates would be greatest near sites at higher risk for

260 exposure to livestock or human feces, compared to sites designated as lower risk for these

261 sources. This hypothesis was supported by our risk factor analysis, in which detection of any of

262 these bacteria was greatest in invertebrates collected near areas at higher risk for exposure to

263 human sewage. However, no significant association was observed between detecting the

264 targeted bacteria and invertebrates collected from areas at higher risk for exposure to livestock

265 runoff, nor were either of the higher risk fecal categories significant risk factors in the logistic

266 regression models for individual bacteria. Study sites considered at higher risk for human

267 sewage exposure included sites near human sewage treatment outfalls, as well as sites influenced

268 by leaking septic tanks. In addition, other non-point sources such as urban runoff could be

269 transporting human and animal fecal bacteria from land to sea in these higher risk areas but could

270 not be assessed in this study. Detecting potentially pathogenic bacteria in invertebrates exposed

271 to human sewage could be due to increased pathogen input into the marine ecosystem with

272 human waste, but could also be due to increased nutrient inflow that could make environmental

273 conditions more favorable for bacteria to grow. Other studies have previously detected

274 associations between compromised water quality in nearshore marine ecosystems and human

275 sewage inputs (24, 25, 35).

276 Based on our recent studies, we also hypothesized that high freshwater outflow exposure and

277 wet season sampling would be associated with detection of fecal bacteria in bivalves (11, 28, 32).

278 In this study, high freshwater outflow exposure was not significantly associated with detecting

279 bacteria in mussels, and while C. *perfringens* was detected most often in the wet season, *Vibrio*

280 spp. and *P. shigelloides* were detected most often in the dry season. These findings suggest that

281 the ecology of bacteria in coastal ecosystems is quite different than the ecology of fecal-borne

282 protozoa. All of the bacteria in this study can be shed in the feces of humans and animals, but

283 unlike the protozoa, many bacteria also survive and grow in environmental reservoirs when the

284 conditions are favorable (9, 18). In other studies, *Vibrio* and *Plesiomonas* spp. have been

285 detected most often during the warmer temperatures of summer and under certain salinity ranges

286 (6, 18, 44). Furthermore, *Vibrio* spp. have been shown to play a role in chitin degradation,

287 biofilm formation, and microbial competence in aquatic ecosystems, again highlighting their

288 specialized ecologic niche in aquatic ecosystems (2).

289 *Clostridium perfringens* was not associated with the same environmental parameters as the

290 other bacteria targeted in our study or others (4, 9). Several studies have detected *C. perfringens*

291 throughout the year under a variety of conditions, and it has been utilized as an indicator for fecal

292 source tracking studies (1, 25, 35). *Clostridium perfringens* is an anaerobic, spore-forming

293 bacteria shed in the feces of a variety of animals and humans, with some genotypes associated

294   with clinical disease (34). The finding that both *Vibrio* spp. and *C. perfringens* were

295   significantly associated with mussels collected when no precipitation had occurred in the

296   previous day suggests that rain may influence the bacteria-invertebrate interactions, possibly by

297   altering the bacterial concentration in surface waters, and/or the invertebrate feeding and

298   depuration dynamics (33).

299   Several types of *Vibrio* spp. were detected in this study. *Vibrio cholerae* is the most

300   infamous of the *Vibrios*, and while none of the epidemic-causing O1 strains were detected in this

301   study, even non-O1 *V. choleraes* have been associated with clinical disease in humans in

302   California and elsewhere (18, 22, 36). *Vibrio parahaemolyticus* is another potentially pathogenic

303   strain that can cause enteritis, and *V. alginolyticus* is the most widespread environmental strain,

304   causing gastroenteritis or wound infections under opportune conditions (3, 18). The current

305   study did not test bacterial isolates for virulence-associated factors to more definitively evaluate

306   their potential pathogenicity, nor did it quantitate bacterial concentrations, another important

307   consideration when specifically evaluating microbial disease potential (5).

308   *Vibrio cholerae* was first reported along the California coast in the 1980's (21-23). The

309   Kenyon et al. (22, 23) studies were undertaken as a follow-up to a human case of *V. cholerae*

310   non-O1 gastroenteritis, and the highest *V. cholerae* levels were detected in water samples during

311   the summer, at times when coliform counts exceeded the legal limit of 1,000 per 100 ml seawater.

312   The Kaysner et al. (21) study tested water, sediment, and shellfish samples for *V. cholerae* from

313   24 estuaries along the U.S. Pacific coast. *Vibrio cholerae* non-O1 was detected in Washington,

314   Oregon, and California, with *V. cholerae* O1 Inaba reported in three water samples from Morro

315   Bay, CA. Predominantly non-O1 *V. cholerae* strains have also been reported on the gulf and east

316   coasts of the United States (20, 46).

317   Two serotypes of *Salmonella* were detected in invertebrates during the wet season collections.

318   *Salmonella* Heidelberg was cultured from fat innkeeper worms living in the muddy estuary floor

319   and *S.* Typhimurium was detected in Pismo clams from the sandy beach just outside the estuary

320   mouth. Both of these *Salmonella* serotypes have been associated with clinical disease and are

321   shed in the feces of a variety of hosts (17, 38, 45). Because *Salmonella* spp. were not cultured

322   from mussels collected at the same time as the benthic invertebrates, it is uncertain whether the

323   *Salmonella* were passing through in the water or were already present in the benthic sediment

324   when filtered by the invertebrates. For most of the other bacterial species, suspended mussels and

325   adjacent benthic invertebrates were culture-positive at the same sampling point, suggesting that

326   the bacteria were passing through in the water column.

327   The comparative invertebrate and freshwater clam surveys showed that many of the same

328   bacterial species isolated from marine mussels were also detectable in upstream invertebrates,

329   though increased sample sizes and source tracking are needed to evaluate these associations

330   statistically. Both estuaries in the study are at the interface between human activities and natural

331   ecosystems, draining watersheds impacted by a wide range of agricultural and urban activities.

332   Both have had problems with pollution and water quality, though invertebrates from both areas

333   are still consumed by humans and marine mammals, such as the federally-listed threatened

334   Southern sea otter (http://www.waterboards.ca.gov/centralcoast/BasinPlan/Documents/3Bacti

335   ObjsStaffReport05-04-04.doc). More invertebrates were positive for bacteria from the muddy

336   sites inside the estuaries than from the sandy sites just outside the estuaries. This finding is

337   consistent with sources of bacteria coming from inland waterways and draining out into the

338   ocean. However, it is also possible that the different environmental conditions inside the estuary

339   are simply more favorable for bacterial survival than outside the estuary, allowing for bacteria

340   that enter the estuary on the incoming tide to find environmental niches in muddy habitat that

341   aren't available in sandy habitat.

342   Mussels (*Mytilus* spp.) and freshwater clams (*Corbicula* spp.) were used for outplanting as

343   sentinel bivalves for specific reasons. First, they already existed in the ecosystems of interest

344   along the California coast, and they had been used for years by coastal monitoring programs to

345   study pesticide and metal pollutants in coastal ecosystems (41). Second, the inherent filter-

346   feeding activities of bivalves make them a natural concentrating mechanism that is easier to

347   process in the laboratory than large volumes of water. Other invertebrates worth considering for

348   sentinel studies include fat innkeeper worms in muddy habitats, that were recently shown to

349   retain much higher levels of the algal neurotoxin domoic acid than other invertebrates along the

350   California coast (http://seafloor. csumb. edu/publications/capstones/goldbergthesis.pdf), and sand

351   crabs that live on sandy beaches where mussels are not always found, and have been shown to

352   retain high levels of domoic acid in recent studies (14).

353   The culture methods used in this study were chosen because they are widely accepted and

354   cost-efficient compared to molecular methods. The use of selective media allowed for efficient

355   sample screening of a large number of samples, followed by further biochemical and molecular

356   confirmation techniques on a more limited sample set. As has been shown in prior studies (10,

357   30, 31), digestive gland was the most sensitive tissue for screening purposes. It is possible that

358   some samples falsely tested negative by culture in our study due to differences between the

359   selective culture conditions and natural environmental conditions, or if the bacteria were in a

360   viable but non-culturable state (15).

361   In conclusion, this study assessed a number of invertebrate species for potentially pathogenic

362   bacteria present in coastal California ecosystems. The increased detection of bacteria in

363   invertebrates exposed to human sewage sources suggests that anthropogenic changes to the

364   nearshore marine ecosystem may have significant effects on the ecology of fecal bacteria at the

365   land-sea interface. Other studies support this association (11, 13, 26), making research on

366   methods to minimize the impacts of humans and their associated animals on nearshore

367   ecosystems important for long-term sustainability and health (1, 7, 42).

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500   Table and Figure captions:

501

502   Table 1. Prevalence of fecal bacteria in mussel batches, 2001-2004.

503

504   Table 2. Univariate analysis of factors significantly associated with detection of enteric bacteria

505   in mussel batches.

506

507   Table 3. Multivariate analysis of factors significantly associated with detection of enteric

508   bacteria in mussel batches.

509

510   Table 4. Bacteria cultured from invertebrate species living in two muddy and sandy estuarine

511   habitats in the wet season, 2002-2003.

512

513   Table 5. Bacteria cultured from freshwater clams in two coastal California Rivers, 2002-2004.[a]

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515   Figure 1. Map of the central California coast showing invertebrate field sampling sites.

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524   Table 1

Bacterial species	Seasonal prevalence(%)		Overall prevalence (%) (n=46)
	Dry (n=27 batches)	Wet (n=19 batches)	
Campylobacter	0	0	0
E.coli-O157	0	0	0
Salmonella	0	0	0
Plesiomonas shigelloides	4	0	2
Vibrio parahaemolyticus	11	5	9
Vibrio cholerae	26	11	20
Vibrio alginolyticus	48	26	39
Clostridium perfringens	41	68	52
Any of the above bacteria	81	79	80

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527   Table 2

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Univariate risk factors	Vibrio cholerae		Vibrio alginolyticus		Clostridium perfringens		Any bacterial species	
	Odds ratio	P value	Odds ratio	P value	Odds ratio	P value	Odds ratio	P value
Fecal risk category - lower	1	-	1	-	1	-	1	-
higher - livestock sources	3	0.2	0.5	0.4	1.4	0.7	2.6	0.4
higher - human sources	3.7	0.3	2.4	0.3	2.1	0.4	13.6	0.08[a]
Season: Dry (June-November)	1	-	1	-	1	-	1	-
Wet (December-May)	0.3	0.2	0.4	<0.01[a]	3.2	0.04[a]	0.9	0.7
Precipitation in the past day: no	1	-	1	-	1	-	1	-
yes	0.4	0.3	0.3	0.1[a]	0.3	0.1[a]	0.1	0.01[a]
Precipitation in the past week: no	1	-	1	-	1	-	1	-
yes	0.6	0.3	0.2	<0.01[a]	0.9	0.8	0.3	0.02[a]
Water temperature over 12°C: no	1	-	1	-	1	-	1	-
yes	1.7	0.5	3.2	0.07[a]	0.3	0.1[a]	0.8	0.6
Water type: estuarine	1	-	1	-	1	-	1	-
marine	0.5	0.5	3.8	<0.01[a]	0.9	0.8	0.3	0.3
Freshwater outflow exposure: low	1	-	1	-	1	-	1	-
medium	1.8	0.4	1.6	0.5	1	1	0.5	0.4
high	1.8	0.4	0.5	0.5	1.7	0.5	0.5	0.5

531   [a]P value <0.1 considered significant.

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535   Table 3

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Bacterial species	Significant risk factor	Odds ratio	P value[a]
Vibrio alginolyticus	Precipitation in the past week: no	1	-
	yes	0.2	<0.01
Clostridium perfringens	Precipitation in the past day: no	1	-
	yes	0.15	0.07
	Water temperature over 12°C: no	1	-
	yes	0.17	0.02
Any bacterial spp.	Fecal risk category: lower risk	1	-
	higher risk - livestock sources	6.9	0.2
	higher risk - human sources	39.4	0.01
	Precipitation in the past day: no	1	-
	yes	0.04	<0.01

539   [a]P value <0.1 considered significant.

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541   Table 4

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Site	Host species	Salmonella[b]	Vibrio parahaemolyticus[b]	Vibrio alginolyticus[b]	Clostridium perfringens[b]
Moss Landing (mud)	Innkeeper worms	+	-	+	+
	Gaper clams	-	-	+	+
	Washington clams	-	-	+	+
	Bent-nose clams	-	-	-	+
	Mussels	-	-	+	+
Moss Landing (sand)	Pismo clams	+	-	-	-
	Blepharipoda crabs	-	-	-	-
	Emerita crabs	-	-	+	-
Morro Bay (mud)	Innkeeper worms	-	-	-	-
	Bent-nose clams	-	-	+	+
	Mussels	-	+	+	+
Morro Bay (sand)	Pismo clams	-	+	+	+
	Blepharipoda crabs	-	-	+	-
	Emerita crabs	-	-	-	-
	Mussels	-	-	+	+

[a]No *Campylobacter* spp., *E.coli* O157:H7, *Plesiomonas shigelloides,* or other *Vibrio* spp. were detected.

[b] + = culture positive batch; - = culture negative batch.

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549   Table 5

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Riverine Study Site	Vibrio cholerae			Vibrio alginolyticus			Clostridium perfringens		
	D1[b]	D2	W2	D1	D2	W2	D1	D2	W2
San Lorenzo Upper	-	-	-	-	-	-	-	-	-
San Lorenzo Middle	-	-	-	+	-	-	+	-	-
San Lorenzo Lower	-	+	-	+	-	-	-	-	-
Salinas Upper	-	+	-	-	-	-	-	-	-
Salinas Lower	-	+	-	+	-	-	-	-	-

[a] No *Campylobacter, E.coli* -O157, *Plesiomonas, Salmonella* , other *Vibrio* spp. were detected.

552   [b] D = Dry season, W = Wet season; 1 = Year 1, 2 = Year 2.